FURTHER STUDIES ON THE ANAEROBIC METABOLISM OF SOME FRESH WATER SNAILS [1]

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It has been shown (von Brand, Baernstein and Mehlman, 1950) that different species of fresh water snails show considerable differences in their resistance to anaerobic conditions. All species consumed carbohydrate anaerobically, but in contrast to what is known from mammalian tissues, lactic acid proved to be only a minor end-product of anaerobic carbohydrate breakdown in several species. It therefore appeared of interest to search for other metabolic end-products and in the present communication data are presented on the formation of volatile acids. This phase was selected because lower fatty acids have been found among the anaerobic metabolic end-products both of insects and worms (review of the literature in von Brand, 1946).

Material and Methods

The following species of snails were employed and where no further data are supplied, they were of the same derivation as stated previously (von Brand, Baernstein and Mehlman, 1950).

1. Pulmonates
   Planorbidae: *Australorbis glabratus, Helisoma duryi, Planorbarius corneus, Biomphalaria boissyi, Biomphalaria pfeifferi*
   Lymnaeidae: *Lymnaea palustris, Lymnaea natalensis*
   Physidae: *Physa gyrina, Aplexa nitens*
2. Operculates
   Thiaridae: *Melanoides tuberculatus*
   Amnicolidae: *Bulimus tentaculatus,* used shortly after being shipped from Lake Erie, Michigan

The general plan of the experiments was to incubate the snails anaerobically for the maximal period compatible with their anaerobic tolerance, to determine the carbon dioxide production during the period of incubation, and to establish at the end of this period whether any volatile acids had been excreted into the medium or accumulated in the tissues of the snails.

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The experimental procedure employed in anaerobic incubation and in determining the carbon dioxide production has been described previously (von Brand, Baernstein and Mehlman, 1950) and the same temperature, 30° C., was used. At the end of the experiments 1 ml. of the medium (dechlorinated tap water), *i.e.,* one-half the amount used initially, was withdrawn from each Warburg flask and the steam volatile acids were determined according to the procedure of Bueding (1949). The snails of each flask were immediately transferred to 0.5 ml. of 0.1 *N* NaOH and homogenized; 2.5 ml. of 0.45 per cent $ZnSO_4$ were added and adjusted to a total volume of 4 ml. The material was therefore deproteinized essentially according to Hagedorn and Jensen (1923). After centrifugation one-half of the supernatant was used for determination of the steam distillable acids.

After these series were concluded, attempts were made to identify the volatile acids. For these experiments only two species of snails, *Australorbis glabratus* and *Helisoma duryi,* were employed. As much medium as possible was removed from the flasks and the contents of six flasks were combined. Similarly, the snails of six flasks were used together and in some instances, as indicated, the medium and the snails were combined. The acids recovered by steam distillation were separated on silica gel columns according to the procedure of Ramsey and Patterson (1945). Confirmation of the chromatographic evidence was sought by applying to the separated acids some of the microchemical tests described by Klein and Wenzl (1932).

Results

The rate of carbon dioxide production of the various species of snails in most instances was in fairly good agreement with the values reported previously (von Brand, Baernstein and Mehlman, 1950), but in a few cases, especially *Lymnaea natalensis* and *Biomphalaria boissyi,* greater differences were obtained. No definite explanation can be suggested; it is possible that differences in size or possibly feeding conditions in the aquaria prior to the experiments played a role.

Insofar as details of the carbon dioxide production are concerned, data on two sets each of *Physa gyrina* and *Helisoma duryi* are presented in Figure 1. They are representative for the non-resistant and resistant group, respectively. It is obvious that the carbon dioxide production proceeded at a fairly regular, though slowly declining, rate. This is a significant point to which we will return in the discussion.

It is very probable that the carbon dioxide values presented are minimal values, since it appears likely that some carbon dioxide retention took place. It is obviously impossible to study this question with complete snails; their calcareous shells make such experiments impossible. We tried to approach the problem by homogenizing snails (*Australorbis glabratus*) after removing them from their shells and comparing the amounts of carbon dioxide liberated into the gas phase of Warburg vessels by a definite amount of lactic acid added through the sidearm with those liberated by the same amount of acid from a bicarbonate solution. We did find that the snail tissues retained per gram tissue about 200 $mm.^3$ carbon dioxide. We are not convinced, however, that this figure is correct; snail homogenates show a pH of about 8.3. It is by no means certain that the same pH would prevail in the tissues of intact snails, since it is possible that during the homogenizing process a loss of carbon dioxide occurs. There seems, at present, no practical way of eliminating this obvi-

ous source of error. In view of this situation we do not present this phase of our experiments in detail. It may be mentioned that the tissues of Australorbis have a considerable alkaline reserve; one gram of tissue liberated upon acidification to pH 3.9 about 2270 mm.^{3} of carbon dioxide.

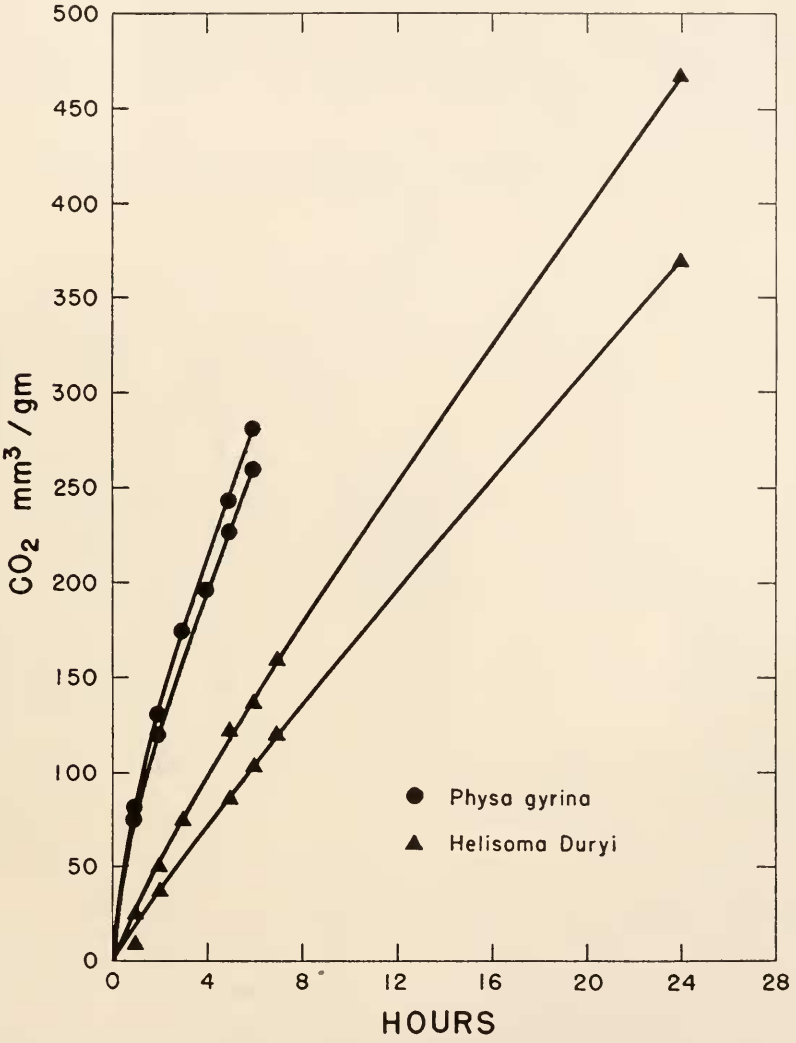

FIGURE 1. Carbon dioxide production of *Physa gyrina* and *Helisoma duryi* in relation to the length of the anaerobic period.

It was established that the snails did not have volatile acids in their tissues before they were subjected to anaerobic conditions. Control determinations with snails taken directly from the aquaria were done with each species and yielded completely negative results. After being exposed to anoxic conditions, on the contrary, significant amounts of volatile acids were found in every case both in the medium and

within the tissues (Table I). It should be noted that the species with little anaerobic resistance (*Aplexa nitens, Physa gyrina, Lymnaea palustris* and *Lymnaea natalensis*) retained a somewhat larger percentage of the acids in their tissues than the resistant ones. In most of the latter surprisingly close agreement was found, the retained acids varying only between 32 and 39 per cent of the total acids produced. *Melanoides tuberculatus* retained only 22 per cent. This snail is the species best adapted to anaerobic survival of all the species used in the present investigation, as the data on anaerobic survival presented by von Brand, Baernstein and Mehlman (1950) indicate.

The volatile acids are unquestionably a major source of the manometrically determined carbon dioxide. In the non-resistant species they are responsible for the evolution of 21 to 40 per cent of the total carbon dioxide, while the corresponding

TABLE I

CO_2 and volatile acid production of fresh water snails under anaerobic conditions

Species	No. of exper.	Anaerobiosis hours	Total CO_2 mm.³/gm.	Volatile acid in tissue % total acid	Volatile acid in medium % total acid	Total volatile acid in ml. of .005 *N*/gm.	% total CO_2 liberated from inorganic source by volatile acid
Aplexa nitens	18	6	381±23	48	52	1.39±0.12	40±2
Physa gyrina	12	6	272±10	46	54	0.78±0.06	32±2.4
Lymnaea palustris	21	6	262±20	56	44	0.50±0.05	21±1.3
Lymnaea natalensis	15	6	400±17	44	56	0.93±0.09	26±2.1
Biomphalaria boissyi	24	16	772±27	36	64	3.76±0.15	55±1.9
Biomphalaria pfeifferi	22	16	402±25	34	66	1.94±0.10	55±1.6
*Australorbis glabratus**	23;30	16	633±29	39	61	3.99±0.19	69±3.3
Helisoma duryi	15	24	436±23	38	62	2.88±0.19	72±2.9
*Helisoma duryi***	17	24	547±24	33	67	4.01±0.18	82±3.1
Planorbarius corneus	13	24	601±50	34	66	3.20±0.34	58±2.8
Melanoides tuberculatus	16	24	140± 8	22	78	0.46±0.06	35±2.8
Bulimus tentaculatus	16	24	255±24	32	68	2.07±0.18	90±3.2

* 23 experiments for the acid production, 30 experiments for the CO_2 production.

** Penicillin and streptomycin in medium.

The figures behind the plus and minus signs represent the standard error of the mean.

range is 55 to 90 in the resistant species. The only exception in this group is again *Melanoides tuberculatus*, with a value of 35 per cent.

The chromatographic separation of the acids showed that two acids were produced both by *Australorbis glabratus* and *Helisoma duryi*, only two distinct bands forming on the silica gel columns. This was established in three separate experiments with the former species in which the medium and the snails were used separately and one experiment in which medium and snails were combined. In the case of *Helisoma duryi*, lack of material prevented a separate study of medium and tissues; two combined samples were investigated. In every instance, the threshold values of the acids corresponded closely to those of acetic and propionic acids and titration of the fractions gave for both species an approximate ratio of 2:1 for the acetic and propionic acid portions, respectively. This was true in the case of Australorbis, both for the medium and the tissues.

The propionic fraction recovered from Australorbis gave with mercurous nitrate

typical crystals of the mercury salt microscopically indistinguishable from those prepared from pure propionic acid. In the case of Helisoma, the copper salt was prepared; the amounts were small, but deep blue crystals characteristic of the copper salts of lower fatty acids were obtained and they were soluble in alcohol. It seems probable, then, that we were dealing in both cases with propionic acid.

The acetic acid fractions gave with mercurous nitrate only non-characteristic crystals. This happens frequently when the slightest traces of impurities are present (Klein and Wenzl, 1932). With copper sulfate deep blue crystals were also obtained and they showed the typical insolubility in alcohol. There can hardly be a doubt, therefore, that we were dealing with acetic acid.

Discussion

The present investigation has shown that snails kept under anaerobic conditions produce volatile acids, which they partly excrete into the medium and partly retain in their tissues, the acids involved being acetic and propionic acids. The formation of the former of these acids is not too surprising since acetic acid is a rather frequent end-product of anaerobic carbohydrate utilization both in bacteria and some higher organisms. Propionic acid, on the other hand, has been found only in the case of Ascaris insofar as multicellular organisms are concerned. Since our snails were not bacteriologically sterile, the question immediately arises whether the fatty acids were actually produced by the snail tissues or were due to bacterial activity which conceivably might have transformed lactic acid derived from the anaerobic metabolism of the snails into the lower fatty acids. It is well known that this question gave rise to a prolonged controversy in the case of parasitic worms. It has recently been decided unequivocally by Epps, Weiner and Bueding (1950), who succeeded in sterilizing *Ascaris lumbricoides* by means of antibiotics and found that these worms did produce lower fatty acids, among them acetic and propionic acid.

We carried out a series of experiments with *Helisoma duryi* kept in dechlorinated tap water containing 2000 units of penicillin and 10,000 units of streptomycin. As the data of Table I show, these snails produced somewhat more carbon dioxide and volatile acid than the nontreated snails, but even this medium yielded upon transfer to a nutritive medium and subsequent incubation some bacterial colonies, and there is no justification to assume that the alimentary tract of the snails would have been sterilized. We abandoned further attempts to sterilize snails because we gained the impression that prolonged stay in the antibiotic solutions, though not killing the snails, affected them adversely.

The Australorbis used in the chromatography experiments were laboratory reared, while the Helisoma were freshly collected from large tanks in Kenilworth Gardens, Md. Both nevertheless yielded the same acids; if bacterial activity was involved, both species would presumably have had to carry the same bacterial flora. Both species also accumulate acids in their body. The internal tissues of snails are presumably sterile; one would then have to assume that the metabolic end-products would have been transported first to contaminated organs, the alimentary tract and perhaps the outside tissues, and have been transformed there to fatty acids. These assumptions, although not impossible, are none too probable. It should also be remembered that the carbon dioxide production of the anaerobic snails declined somewhat with time; if a bacterial population would have been built up, one would have

expected rather an increase in rate. We are inclined to assume tentatively that the acids originated from the metabolism of the snails, but we concede that the final decision will have to wait for work on bacteriologically sterile snails.

We wish to emphasize strongly, however, that the biological implications of our findings are unaffected by the question of whether the snails themselves produced the volatile acids or not, since in nature no sterile snails can be expected to occur. We have pointed out previously (von Brand, Baernstein and Mehlman, 1950) that the non-resistant species, in contrast to the resistant ones, accumulated lactic acid within their tissues and we assumed that this difference may be one of the factors explaining the difference in resistance to anaerobiosis. We pointed out, however, that lactic acid could not be responsible alone for the death of the snails, because the resistant species also ultimately died of asphyxiation. Our present results would seem to indicate that the marked accumulation of volatile acids in the tissues may

TABLE II

Percentage of anaerobic carbon dioxide liberated from inorganic sources

Species	Per cent of total carbon dioxide liberated from inorganic sources		
	By lactic acid*	By volatile acids	Total
Aplexa nitens	67	40	107
Physa gyrina	38	32	70
Lymnaea natalensis	139	26	165
Lymnaea palustris	41	21	62
Biomphalaria boissyi	25	55	80
Biomphalaria pfeifferi	17	55	72
Australorbis glabratus	13	69	82
Helisoma duryi	4	72	76
Planorbarius corneus	3	58	61
Melanoides tuberculatus	17	35	52

* Data from von Brand, Baernstein and Mehlman (1950).

have a bearing on the problem. The lower fatty acids are probably less toxic than lactic acid, but still if they accumulate to a certain level they may be harmful. The difference in the respective amounts of end-products of various toxicities within the tissues of different species of snails may well be at the root of the differences in anaerobic resistance. Whether it is the sole factor involved cannot be stated with any degree of certainty, however.

It would obviously be premature to speculate about the mode of formation of the volatile acids, but a brief discussion of the carbon dioxide picture seems appropriate. We have pointed out in our previous paper that the carbon dioxide evolved by the non-resistant species is largely of direct inorganic origin, that is, liberated by lactic acid from bicarbonate. The relevant data concerning this point are shown again in Table II. The third column shows the amounts of carbon dioxide liberated from inorganic sources by the volatile acids. It is now clear that in all species a very large percentage of the carbon dioxide is of direct inorganic origin. It is possible or even probable that the remainder of the carbon dioxide unaccounted from this source is true respiratory carbon dioxide, carbon dioxide derived from the break-

down of carbohydrate. No accurate data along this line can be calculated, however, because of our inability to elucidate adequately the question of carbon dioxide retention by the tissues. It may be pointed out that this source of error may be responsible for the fact that less carbon dioxide was found in the cases of *Lymnaea natalensis* and *Aplexa nitens* than corresponds to the acids produced.

Summary

1. Fresh water snails exposed to anaerobic conditions produce volatile acids which are partly excreted into the medium and partly accumulate in the tissues.

2. The acids formed by *Australorbis glabratus* and *Helisoma duryi* were identified by chromatographic means and crystallographic data as propionic and acetic acids.

3. While bacterial formation of these acids cannot be excluded categorically, some evidence is adduced to the effect that they may be produced by the snail tissues.

4. The evidence indicates that the species not resistant to anaerobiosis are killed primarily by the accumulation of lactic acid, while the resistant species are more tolerant to the lack of oxygen due to the fact that they accumulate in their tissues the less toxic fatty acids rather than lactic acid.

5. Most of the carbon dioxide evolved by anaerobically kept snails is of direct inorganic origin.

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